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Fluorescence polarization immunoassay of colchicine

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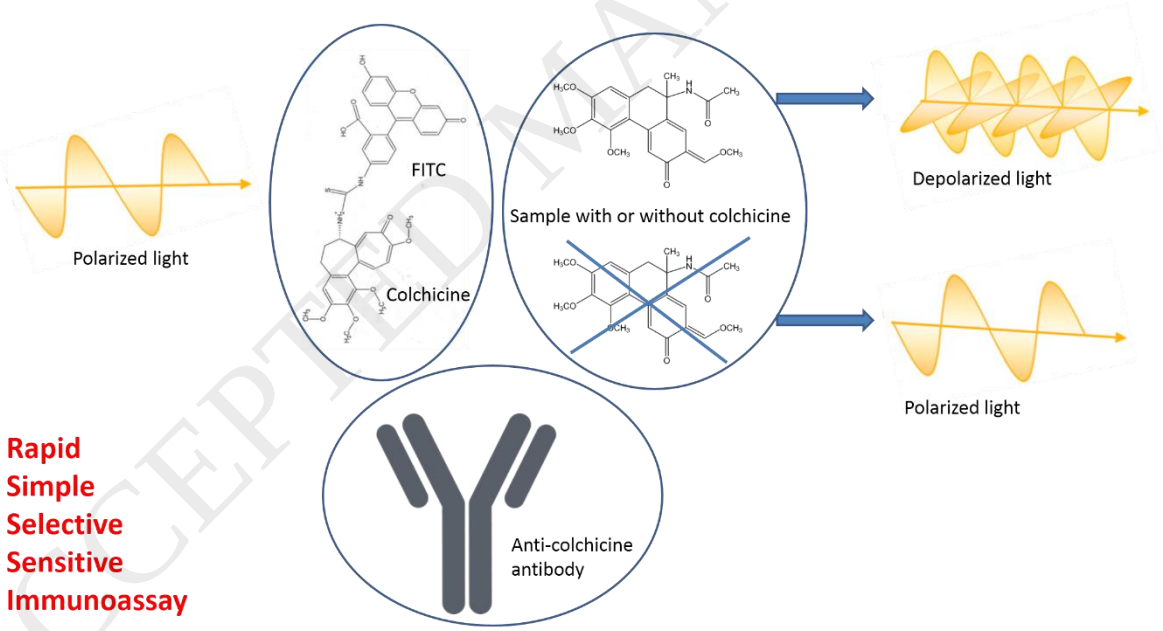
Graphical abstract



Highlights

- Fluorescence polarization immunoassay was developed to detect colchicine
- Duration of the analysis was 10 min
- Detection limit of the analysis was 1.8 ng/mL
- Applicability of the assay for control of drug formulations, urine, milk was confirmed

Abstract

In this study, a fluorescence polarization immunoassay (FPIA) technique was developed to determine colchicine (COL), an alkaloid of noxious plants of the order *Liliales* that is used in a number of medications to treat gout. An optimal combination of the polyclonal antibody and the antigen labelled with fluorescein isothiocyanate (FITC) was selected. Conditions for the competitive interaction of the antigen in the tested samples and its fluorophore conjugate (COL–FITC) with anti-COL antibodies were optimised, and the analytical characteristics of the assay were determined. The developed FPIA was characterised by a detection limit of 1.8 ng/mL and a detectable analyte concentration range of 4.1–74.3 ng/mL. The duration of the analysis was 10 min. The applicability of the developed FPIA for quality control of ready-made drug formulations and for the estimation of COL content in various matrices (urine, milk), with recovery values ranging from 79–108%, was demonstrated.

Keywords: fluorescence polarization immunoassay, colchicine

1. Introduction

Colchicine (COL) ((S)-N-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl)acetamide) is a tropolonic alkaloid isolated from plants of the genus *Colchicum* (usually called *Colchicum autumnale*) and from *Gloriosa superba*. Since ancient times, herbal extracts containing COL have been used in folk medicine to treat gout [1]. In modern medicine, COL prevents gout and gouty attacks, and it is used as the preferred agent in the treatment of acute gouty arthritis, familial Mediterranean fever, etc. [1-3]. However, COL is known to display a genotoxic effect (as a mitosis inhibitor); its uptake can result in a variety of side effects even when the recommended dosage is observed [4, 5]. High doses of COL can cause acute poisoning and lead to severe systemic toxicity (a dose of 0.8 mg/kg or more usually leads to cardiogenic shock) [4, 6].

Therefore, the use of COL-based medicinal preparations to treat musculoskeletal system diseases must be monitored by continual control of the COL concentration in the patient [1, 7]. This requirement necessitates the development of new effective test methods that allow for the precise and rapid determination of the COL concentration in biological fluids, such as blood and urine [8] (up to 65% of orally administered COL is recovered unchanged in urine [9]). Moreover, the determination of the concentration of COL is relevant to ensure quality control of ready-made drug formulations to reveal counterfeit products, the appearance of which on the pharmaceutical market is stipulated by the high cost of COL-containing drugs. Since COL is

known to be used in veterinary medicine to treat papillomas and warts in cattle and horses, and it can also enter the body of animals via feed [10, 11], it is necessary to determine the concentration of COL in environmental objects and foodstuffs. Due to the serious health risks associated with the uptake of food products contaminated by COL, this substance is included in Annex IV Council Regulation (EEC) No 2377/90 [12]. That regulation specifies that the administration of the substances listed in Annex IV to food producing animals should be prohibited.

To date, liquid chromatography methods with different types of detection are commonly used to determine the concentration of COL in various samples (biological liquids, pharmaceutical preparations, including those produced from plant raw materials, food matrixes, etc.). Reversed-phase high performance liquid chromatography [6, 13] and liquid chromatography–tandem mass spectrometry [14-16] are examples of the methods that are commonly used. In addition, high-performance thin-layer chromatographic [13, 17] and electrochemical methods [18] and capillary electrophoresis [19] have been proposed for this purpose. Although chromatographic analysis is characterised by a high accuracy of the analyte measurement, its implementation requires using expensive equipment and multi-stage sample preparation techniques, which significantly increases the duration and cost of a single test. In this regard, immunochemical methods that combine high sensitivity and specificity are in great demand for solving the problems associated with high-performance screening. They can be conducted using relatively simple instrumentation, they do not require complex clean-up procedures and they allow for simultaneous analysis of up to hundreds of probes.

Radioimmunoassay (RIA) is mainly proposed for the immunodetection of COL [20, 21]. However, widespread use of the RIA is hampered by a number of limiting factors, including the need for premises specially equipped to work with radioactive isotopes, rather complicated instrumentation and the destruction of the biomolecules under the influence of many long-lived isotopes. Therefore, to date, immunoanalytical methods that are suitable for mass monitoring of samples for COL content are not available. It should be noted that a transition to rapid analytical approaches that allow obtaining results within 10–15 min is a predominant trend in modern immunodiagnostics [22, 23].

A fluorescence polarization immunoassay (FPIA) can be proposed as one of the most promising solutions for express immunodiagnostics [24]. FPIA is a homogeneous method based on the competition between the antigen in the sample and the antigen–fluorophore conjugate for the interaction with antibody binding sites. Here, upon antibody binding with a colchicine–fluorescein isothiocyanate (COL-FITC) conjugate, the size of the entire fluorescent complex increases and its mobility decreases, resulting in the substantial growth of fluorescence

polarization (FP). FPIA has several advantages, such as high performance, since it lacks the stages of adsorption of immunoreagents and washing [25] that are characteristic of a heterogeneous immunoassay. Therefore, this study aimed to develop a FPIA to determine COL in various matrices (urine, milk).

2. Materials and methods

2.1. Materials

COL, sodium azide, FITC isomer I, triethylamine, Triton X-100 and Tween-20 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Silicagel plates for thin-layer chromatography (TLC) were obtained from Merck (Darmstadt, Germany). Anti-COL sheep polyclonal antibodies were obtained from MicroPharm (Newcastle Emlyn, UK). All auxiliary reagents (salts, acids, alkalis and organic solvents) were of analytical or chemical purity.

A 3 mM borate buffer, pH 9.0, containing 0.01% (w/v) sodium azide (BB) or a 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM NaCl (phosphate-buffered saline [PBS]) were used to dilute the immunoreagents. Milli-Q deionized water (Merck, Darmstadt, Germany) was used to prepare a stock solution of COL (1 mg/mL).

MaxiSorp black microplates (Thermo Fisher Scienific, Roskilde, Denmark) were used for the FPIA. Fluorescence polarization was measured on the Zenyth 3100 (Anthos Labtec Instruments, Wals-Siezenheim, Austria) microplate photometer. The excitation wavelength was 485 nm and the emission wavelength was 535 nm.

2.2. Synthesis and purification of the COL–FITC conjugates

A fluorescently labelled COL was synthesised using FITC and a derivative of COL previously obtained as a product of its amide bond hydrolysis (COL–NH_2). Standard protocol for FITC conjugation with haptens was used [26]. For this purpose, COL–NH_2 (2 mg) and FITC (2 mg) were dissolved in 0.5 mL of methanol, and then 50 µL of triethylamine was added. The obtained mixture was incubated for 12 h at room temperature with stirring. The COL–FITC conjugates were isolated by TLC conducted in the chloroform–methanol mixture (1:1, v/v). The yellow fluorescent bands were collected from the plate, and the conjugates were extracted with 1 mL of methanol. The purified conjugates were stored at 4°C. The success of syntheses and structures of tracers were confirmed by high-resolution tandem mass spectrometry coupled with high-performance liquid chromatography (HPLC-MS/MS).

2.3. Selection of COL–FITC working dilution for the FPIA

Briefly, 100 μL of the COL–FITC conjugate in BB (serially diluted from 1:100 to 1:100 000) was added to the microplate wells. Then, 100 μL of BB was added to each well and the FP was measured.

2.4. Selection of the working dilution of the antibodies for the FPIA

First, 100 μL of the anti-COL antibodies at several dilutions, ranging between 1:200 and 1:25600, in BB were added to the microplate wells. Then, 100 μL of the COL–FITC conjugate in the working dilution was added to the wells. The resulting solution was stirred for 5 min, and then the FP was recorded.

2.5. FPIA of COL

Standard solutions of COL (in concentrations ranging from 0.1 ng/mL to 10 μg/mL in BB) were prepared to obtain a calibration curve. For the assay, 20 μL of the standard solution was mixed with 100 μL of the COL–FITC conjugate in the working dilution and 100 μL of the anti-COL antibodies in the working dilution. The resulting solution was stirred for 5 min, and the FP was recorded.

2.6. Registration and processing of the assay data

Based on the FP (y) of the analyte in various concentrations (x), a calibration curve was constructed using the four parameter sigmoid function [27]:

$$y = (A–B) / (1 + (x/C)^D) + B,$$

where x is the analyte concentration, y is the FP value, A is the asymptotic maximum of the FP value, B is the asymptotic minimum (background value) of the FP value, C is the inflection point of the curve in the semi-logarithmic coordinates (equal to 50% inhibition of the intensity) and D is the slope of the curve at the inflection point.

The detection limit was calculated as the COL concentration corresponding to 10% binding inhibition. The working range was calculated as the COL concentration corresponding to 20% binding inhibition (the lower limit of the working range) and 80% binding inhibition (the upper limit of the working range).

2.7. Sample preparation of the pharmaceuticals

Two kinds of pharmaceuticals from different manufacturers were tested. The tablets were dissolved in distilled water to obtain a COL concentration of 1 mg/mL. The resultant solutions were centrifuged for 10 min at 10,600 g. The obtained supernatants were used for the analysis.

2.8. Sample preparation of milk

Milk with a 3.2% fat content was purchased at a local supermarket. The milk samples were spiked with different amounts of COL, and then mixed thoroughly. To prepare the samples, a part of the protein fraction and fats was precipitated using a saturated solution of ammonium sulfate [$(NH_4)_2SO_4$] [28]. For this purpose, 1 mL of milk was mixed with 1 mL of the $(NH_4)_2SO_4$ saturated solution. The resultant mixture was stirred for 5 min, and then centrifuged for 15 min at 10,600 g. Consequently, a layer of fat and sediment on the bottom of the microtube were formed. The liquid under the fat layer was removed, diluted 2 times with PBS and used for the FPIA.

3. Results and discussion

3.1. Selection of the immunoreagents

The proposed detection method is based on the competitive interaction of free COL in the tested samples and the fluorescein-labelled COL with specific antibodies. Specific binding between the COL–FITC conjugate and an immunoglobulin molecule leads to the increase in the dimension of the entire fluorescent complex; this decreases its mobility, which results in the essential growth of the FP. Addition of free COL causes competition, decreasing quantity of the COL-FITC – immunoglobulin complexes and, due to this, lower FP (Fig. 1).

To develop the FPIA, a COL–FITC conjugate (see the structure presented in Fig. 2) was synthesised by the direct interaction of FITC with the COL derivative obtained as a result of the amide bond hydrolysis of the COL molecule. The synthesis produced three fractions, characterised by different retention factor (*Rf*) values The working solution of each conjugate was selected so that its fluorescence intensity was at least 10-times higher than the background signal (i.e. that of BB). Finally, the COL–FITC conjugates that were used in the working dilutions ranged from 1:6000 to 1:8000.

The interaction between the specific polyclonal antibodies (a total of 12 preparations of specific anti-colchicine antibodies) and all three fluorescent conjugates was studied. For this purpose, the antibodies in a series of dilutions were incubated with a COL–FITC conjugate in a previously established working dilution. A pair of "antibody–labelled COL" that demonstrated the highest affinity for the interaction was selected for additional FPIA experiments. The corresponding concentration dependence is presented in Fig.S1.

3.2. FPIA of COL

Optimisation of the competitive FPIA conditions included selection of the antibody concentration and the content of the reaction media. The dependence of the IC_{20} and IC_{50} values on the antibody dilution and the competitive curves of COL obtained using different dilutions of antibodies are presented in Table 1 and Fig. 3, respectively. It was demonstrated that the antibody dilution of 1:10000 provided a stable analytical signal and the maximum assay sensitivity (curve 4 in Fig. 3). Increased dilution of antibodies resulted in a significant decrease in signal stability.

A 3 mM borate buffer, pH 9.0, containing 0.01% sodium azide (BB) was used as the reaction media for the FPIA. An increase in the buffer molarity up to 50 mM did not affect the results of the measurements. In our previous study, we found that the replacement of BB with PBS in the FPIA of ractopamine (an agonist of beta-adrenoreceptors) resulted in a significant increase in the maximum FP signal and shortened the assay duration. In the case of the FPIA of COL, replacing the borate buffer with PBS led to a significant increase in the stability of the analytical signal, resulting in the reduction of standard errors. The observed pH-dependent effects could be potentially caused by pH-dependence of FITC fluorescence and its ability to form dimers and other aggregates, which affects precisely the reproducibility of the results obtained [29]. The addition of Triton X-100 detergent to PBS did not prove to be effective since it resulted in a significant reduction in the ΔmP value and it decreased the sensitivity of the assay.

Therefore, the optimal working dilutions of the COL–FITC conjugate (Rf = 0.8) and anti-COL antibodies were 1:6000 and 1:10000, respectively. To perform a competitive FPIA, 20 μL of a COL solution and 100 μL of the COL–FITC conjugate, 1:6000, were added to the microplate wells. After addition of 100 μL of the anti-COL antibodies, 1:10000, and incubation, the FP was measured. The study of the association kinetics of the COL–FITC conjugate with the antibodies and the subsequent displacement of this conjugate from the immune complex with free COL showed that the sensitivity of the assay conducted in this mode was comparable to that of the traditional competitive assay only if the displacement step lasted 60 min. This effect makes this type of assay scheme unreasonable. Fig. 5 shows the calibration curve for the FPIA of COL obtained in the optimised conditions.

The developed FPIA was characterised by the detection limit of COL of 1.8 ng/mL and the detectable concentration range of 4.1–74.3 ng/mL.

3.3. Analysis of the contaminated samples

Before evaluating the applicability of the developed FPIA for quality control of the ready-made drug formulations and for its ability to detect COL in various matrices (urine, milk), the requirements for sample preparation were considered.

Analysis of the pharmaceuticals. The pharmaceuticals were tested immediately after their dissolution in water. This led to a significant decrease in the analytical signal because, upon dissolving a tablet, a suspension consisting of a large amount of fillers and stabilisers (corn starch, lactose monohydrate, microcrystalline cellulose, etc.) was formed. It was found that centrifugation of the sample for 10 min at 10,600 g before testing was the optimal way to eliminate the matrix influence on the assay results.

Analysis of the milk samples. Milk is a complex system consisting of a large amount of proteins and fat, which can affect the immunoassay results. To reduce that influence, the milk samples were preliminarily treated using a concentrated $(NH_4)_2SO_4$ solution.

Analysis of the urine samples. It was demonstrated that complex preliminary sample preparation is not required before testing the urine samples. These samples diluted 2 times with PBS and used for the FPIA.

Taking into account the selected conditions of the sample preparation (see the Materials and methods section), the milk and urine samples were spiked with known quantities of COL, and then analysed by FPIA. The estimated recovery values are presented in Table 2.

Hence, the developed FPIA allowed for detecting 79–108% of COL in real matrices; therefore the obtained results are reliable.

4. Conclusion

This study was the first to develop a FPIA technique to detect COL. FPIA is characterised by a COL detection limit of 1.8 ng/mL and a detectable COL concentration range of 4.1–74.3 ng/mL. The assay duration is 10 min. The applicability of the developed FPIA for quality control of ready-made drug formulations and for the detection of COL in various matrices (urine, milk, pharmaceuticals) was confirmed.

Acknowledgments

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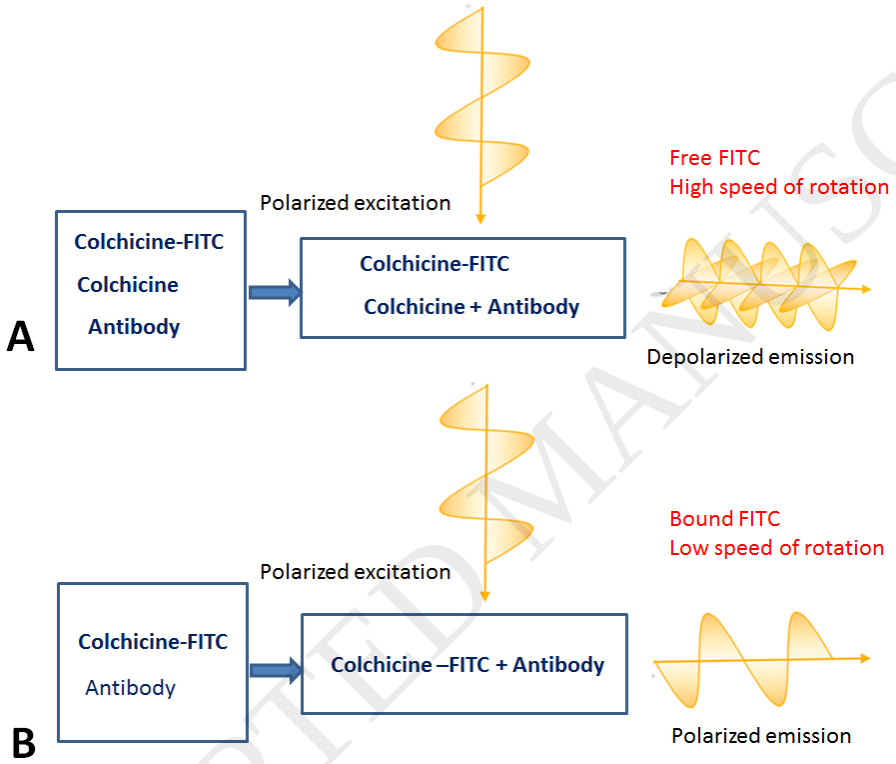
Figure captions

Fig. 1. Scheme of the FPIA. A – for sample with colchicine; B - for sample without colchicine.

Fig. 2. Structural formula of the COL–FITC conjugate.

Fig. 3. Competitive curves for the FPIA of COL. The dilution of the antibodies is 1:7000 (1), 1:8000 (2), 1:9000 (3) and 1:10000 (4) (n = 3).

Fig. 4. Calibration curve for the FPIA of COL in PBS.



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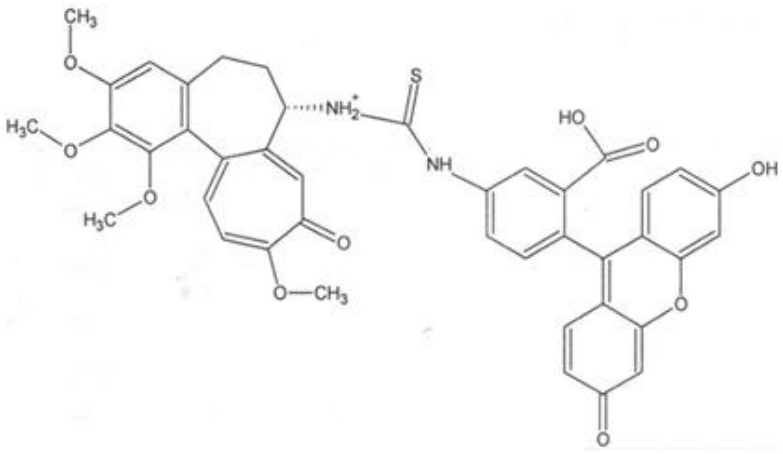

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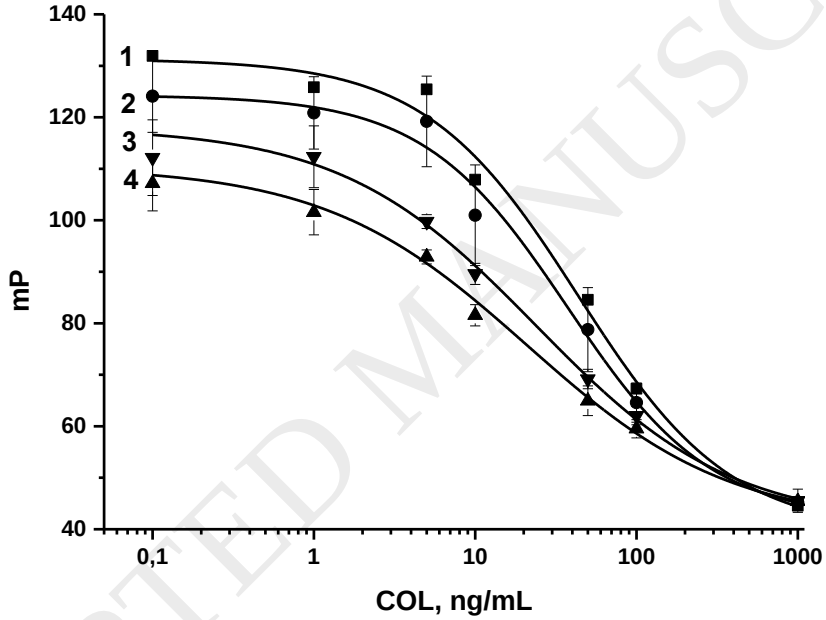

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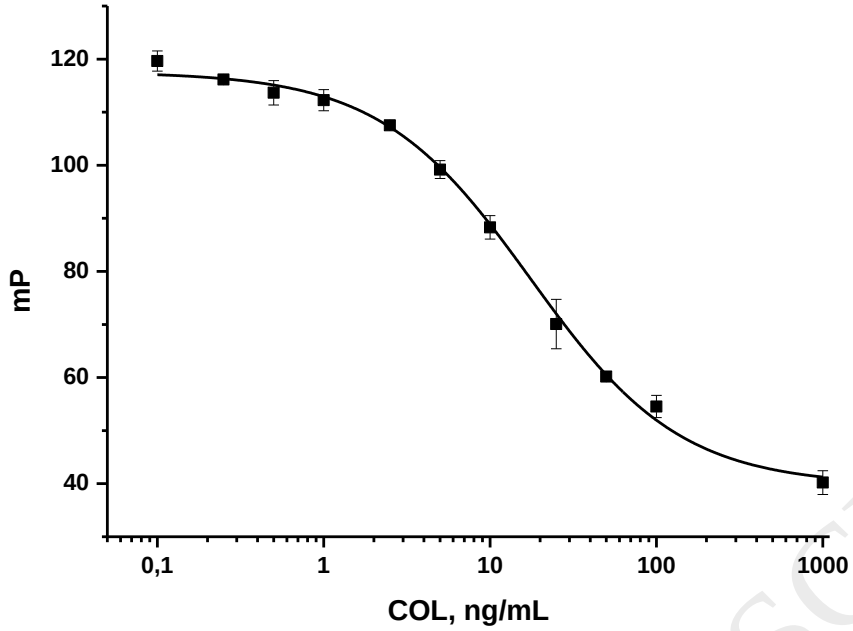

Fig. 4. Calibration curve for the FPIA of COL in PBS.

Table 1. IC_{50} & IC_{20} values obtained in the FPIA based on the dilution of the antibodies. The dilution of the COL–FITC conjugate (*Rf* = 0.8) was 1:6000.

Antibody dilution	IC_{50}, ng/mL	IC_{20}, ng/mL
1:7000	43.3 ± 10.1	9.6 ± 2.4
1:8000	37.4 ± 5.4	9.1 ± 2.3
1:9000	23.9 ± 2.1	3.4 ± 1.1
1:10000	21.1 ± 5.1	3.0 ± 1.4

Table 2. Determination of COL in various matrices using FPIA (n = 3)

Matrix	Added COL concentration, ng/mL	Found COL concentration, ng/mL	Recovery (%)
Anti-gout pharmaceuticals	3	2.8±0.1	92
	10	10.8±1.2	108.5
	50	45.6±5.1	91.3
	100	95.3±11.2	93
Milk	3	-	-
	10	8±1	80

	50	42±5	84
	100	79±8	79
Urine	3	-	-
	10	9.5±0.4	95
	50	48.5±0.3	97
	100	95±3	95